

# Target Organs and Systems: Methodologies to Assess Immune System Function

Meryl H. Karol

Department of Environmental and Occupational Health, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania

Immunotoxicity encompasses both reduced and heightened immune function. Diverse chemicals can impair functioning of the immune system. Both monographs and books have been devoted to detailed descriptions of immunotoxicity. This paper gives a brief overview of the methods currently used to assess the immunotoxic potential of chemicals. It also discusses the trend toward the use of alternative methods to mammalian models, such as feral species, *in vitro* assays, and computational models. The strategy of using a tier approach to screen chemicals for immunotoxicity is described, together with the rationale for, and limitations of, this approach. Interpretation of data with regard to clinical disease and human health is addressed. The immune system poses substantial complexities in this regard as the system has functional reserve and functional redundancy. — *Environ Health Perspect* 106(Suppl 2):533–540 (1998). <http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-2/533-540karol/abstract.html>

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## Introduction

The immune system is a complex, multi-component system that comprises lymphoid organs located throughout the body and interacting, specialized cells. The major lymphoid organs are the spleen, thymus, lymph nodes, bone marrow, and areas of the intestine (Peyer's patches). The major cell type is the lymphocyte; accessory cells include macrophages, natural killer (NK) cells, eosinophils, basophils, dendritic cells, and epithelial cells, among others.

The immune system functions to protect against both the external environment and the internal development of nonself neoplasms. A branch of immunology designated tumor immunology is devoted to the study of the immune responses acquired to tumor antigens and the nature of the antigens on tumor cells that induce the immune responses. Dysfunction of the immune system results

in increased incidence and severity of infection, increased reactivity toward environmental agents (hypersensitivity), and increased development of tumors.

The organs, tissues, cells, and molecules of the immune system interact to provide a coordinated immune response. Lymphocytes circulate among lymphoid organs throughout the body. Through surface receptors, they recognize three-dimensional molecular configurations. Upon engagement of surface molecules with structures having complementary conformation, lymphocytes become activated. They proliferate, yielding generations of cells capable of recognizing and responding to the same antigen upon future encounter (1).

The two main populations of lymphocytes are B lymphocytes and T lymphocytes. Both types of cells function through

recognition of antigens; B cells recognize antigen in its native form, whereas T lymphocytes recognize processed antigen. Encounter of B cells with complementary antigen results in cell activation, differentiation into plasma cells, and secretion of antibodies that recognize the stimulatory antigen. The five main classes of antibody are IgM, IgG, IgA, IgE, and IgD. IgG is found in high concentrations in the blood, approximately 12 mg/ml, whereas IgE is found in very low concentrations, about 20 ng/ml. In general IgA, IgG, and IgM have a protective function by facilitating phagocytosis of particulate antigen and activation of complement. IgE is the major antibody class involved in hypersensitivity reactions.

T cells recognize segments of antigen in association with products of the major histocompatibility complex. There are two main classes of T lymphocytes that differ in function (1). Cytotoxic T cells destroy infected host cells; helper T cells (Th cells) function through production of chemotactic or proinflammatory cytokines. Cytokines are biologically active peptides, produced by a variety of cell types, that modulate the function of cells in immunological reactions including host resistance (HR) and regulation of the immune response. There are two main populations of Th cells. In mice and in humans, cytokines secreted by Th1 cells stimulate a delayed-type hypersensitivity (DTH) response; those released by Th2 cells stimulate IgE production and favor an immediate-onset reaction.

Immunotoxicity can be defined as the adverse effect of chemicals or agents on the immune system. The effect may be increased immune activity, manifested as either hypersensitivity or autoimmunity, or decreased immune function, with reduced ability to combat infectious agents or tumors.

Diverse agents have been associated with immunosuppression in animals, including chemicals, bioaerosols (bacteria, algae, fungi), and physical agents such as ultraviolet light (1). Heightened immune responsiveness, including allergy, may result from chemical exposure. The two most important allergic diseases in the context of occupational and environmental exposures are allergic contact dermatitis (ACD) and respiratory hypersensitivity (RH). ACD is prevalent in certain industries and can be induced by hundreds of diverse chemicals. T lymphocytes are central to the pathogenesis of ACD. RH is caused by relatively few low molecular weight chemicals (i.e., less

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Address correspondence to Dr. M.H. Karol, Department of Environmental and Occupational Health, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, PA 15238. Telephone: (412) 967-6530. Fax: (412) 967-6611. E-mail: mhhk@vms.cis.pitt.edu

Abbreviations used: ACD, allergic contact dermatitis; CMI, cell-mediated immunity; ConA, concanavalin A; DTH, delayed-type hypersensitivity; FEV<sub>1</sub>, forced expiratory volume in 1 sec; HI, humoral immunity; HR, host resistance; LMW, low molecular weight; LPS, lipopolysaccharide; MEST, mouse ear-swelling test; MLR, mixed lymphocyte response; NK cells, natural killer cells; PFC, plaque-forming cells; PHA, phytohemagglutinin; RH, respiratory hypersensitivity; SAR, structure-activity relationship; SRBC, sheep red blood cells; Th cells, helper T cells; U.S. TLV, U.S. threshold limit value.

than 1 kDa) (2), but by numerous high molecular weight environmental agents. Allergic diseases caused by environmental agents are widespread. The incidence in the Western world of one such disease, asthma, is increasing. The reason for the increase is unclear, although environmental agents and delayed exposure of children to childhood viral infections have been suggested as contributing factors (3).

Allergy develops in two stages. Initial encounter with the agent results in a primary immune response and a state of heightened responsiveness to future encounter with that agent. Subsequent exposure of the now sensitized individual to the same allergen results in a secondary immune response that is both more vigorous and accelerated. The secondary response may be a production and release of specific antibodies, cytokines, or other inflammatory mediators to produce inflammation and allergic response. Differences in susceptibility to hypersensitivity relate to the immunologic repertoire of the individual, i.e., the ability of the immune system to recognize and respond to small, structurally simple antigens.

## Tier Approach to Testing

Due in part to the complexity of the immune system, assessment of test chemicals for immunotoxicity utilizes a variety of immune assays. The test methods, as well as the principles supporting the methodology, have been thoroughly described (4). A tiered approach to testing is undertaken that includes examination of pathological changes and assessment of functional activity (5). Assays have been conducted in mice and rats and found to be robust and reproducible. Correlations between test outcomes and altered host defense have been studied (6) to reduce the dependence on multiple assays and to determine the ability of the tests to predict immunologic disease in humans.

### Tier I

Tier I assesses three aspects of immune system integrity: cell-mediated immunity (CMI), humoral immunity (HI), and immunopathology (Table 1). Test chemicals are administered in doses that do not produce overt toxicity.

#### Cell-Mediated Immune System

Cell-mediated immunity is assessed by measurement of the blastogenesis and proliferation of lymphocyte populations when stimulated with nonspecific mitogens (7).

**Table 1.** Tier approach to detecting immune system toxicity in rodents.

<b>Tier I tests</b>
Hematology (e.g., cell counts)
Organ weights
Organ cellularity
Lymphoid organ histology
IgM antibody PFC
Lymphocyte blastogenesis
T-cell mitogens (PHA, ConA)
T-cell mixed leukocyte response
B-cell mitogens (LPS)
Natural killer cell activity
<b>Tier II tests</b>
Splenic B, T cell counts
IgG antibody PFC count
Cytotoxicity T cells; or DTH
HR assay
Syngeneic tumor cells
PYB6 sarcoma (tumor incidence)
B16F10 melanoma (lung burden)
Bacterial models
<i>Lysteria monocytogenes</i> (morbidity)
<i>Streptococcus</i> (morbidity)
Viral models
Influenza (morbidity)
Parasite models
<i>Plasmodium yoelii</i> (parasitemia)

T cells are tested by incubation with phyto-hemagglutinin (PHA) or with concanavalin A (ConA); B cells are tested by incubation with lipopolysaccharide (LPS). These assays are performed by mixing isolated spleen cells from rats or mice with the mitogen in several concentrations. <sup>3</sup>H-Thymidine is added 4 to 18 hr prior to cell harvest. Cells are harvested and radioactivity is assessed. Results are dependent on numerous biochemical events that culminate in DNA synthesis and cell division. Such tests for CMI have been used to assess lymphocyte function in patients with immunodeficiency disease, notably in asymptomatic acquired immunodeficiency syndrome (AIDS) patients.

Another test of CMI is the mixed lymphocyte response (MLR). This assay measures the proliferation of lymphocytes when stimulated by allogeneic cells. Cell proliferation occurs as a consequence of the incompatibility of the allogeneic determinants expressed on the surface of the two lymphocyte populations. The test is performed using murine or rat lymph node cells. Single cell suspensions obtained from mesenteric, inguinal, axillary, and cervical lymph nodes are mixed with stimulator cells that have been treated with mitomycin C. Mixed cultures are incubated and <sup>3</sup>H-thymidine is added as described above (8). Caution is needed to remove all traces of endotoxin from cell preparations.

A third method to assess CMI evaluates NK cell function (9). NK cells are often the first cell type to succumb to immunosuppression by a xenobiotic agent. They have the natural ability to kill tumor cells, virus-infected cells, and autologous monocytes harboring intracellular bacteria. They are effective against fungal invasion by rapidly releasing cytokines that activate and recruit neutrophils. NK cell dysfunction impairs host defenses against viruses, bacteria, fungi, and neoplasms.

Assessment of NK cell function utilizes cells obtained from the peripheral blood and tumor cells previously labeled with <sup>51</sup>Cr as the target cells. In the assay, rodent cells obtained from the blood or spleen are added to microtiter wells containing <sup>51</sup>Cr tumor cells (K562 for human NK activity and YAC-1 for rodent NK activity). After incubation at 37°C for 4 to 6 hr, the supernatant fluid is assessed for released <sup>51</sup>Cr. NK function is expressed as percent lysis at a particular effector cell/target cell ratio.

### Humoral Immune System

Assessment of humoral immunity, included in tier I tests, typically uses the IgM antibody plaque-forming assay. This measures the primary effector function, i.e. the synthesis and secretion of antigen-specific antibodies. This test appears to be the most sensitive of those developed to measure chemical perturbation, and is the most commonly affected functional parameter in animals exposed to chemical immunosuppressants (5).

Mice receive intravenous or intraperitoneal administration of sheep red blood cells (SRBC) (10). Four days later, animals are killed, and the spleen is removed and separated into single cells. The cells are mixed in warmed agar with an aliquot of the SRBC preparation used for immunization and with guinea pig complement. The mixture is then poured into a petri dish or culture plate, covered, and incubated for 2.5 to 3 hr at 37°C. The number of plaque-forming cells (PFC) is counted using a viewer. Calculation is made of the number of PFC per spleen or per 10<sup>6</sup> spleen cells. As described, the assay measures the production of IgM antibody to SRBC. Modifications to the procedure allow measurement of specific IgG or IgA antibody.

### Immune Pathology

Tier I also includes assessment of the immunopathology of key organs of the test animal. Blood, lymph nodes, bone marrow, thymus, spleen, and mucosal-associated lymphoid tissues are examined. Morphology is

noted and various stains are used to detect changes in cellular function. Recent developments include molecular probes to detect DNA damage, *in situ* hybridization to detect gene activation, and magnetic resonance imaging to allow evaluation of the same tissue over a period of time (11).

## Tier II

Compounds that test positive in tier I assays are evaluated further to better define the immunotoxic effect and to assess the effect of the chemical on HR to an infectious agent or tumor. If the results of tier I tests are negative, the chemical is considered not immunotoxic under the conditions and in the dosages used in the screening tests.

The selection of tier II tests is flexible and depends on the nature of the immunologic findings observed in tier I assays (5). Tests frequently used in tier II analysis are given in Table 1. Enumeration of the lymphocyte populations within the spleen (B and T cells) is performed using fluorescent-labeled immune reagents to detect cell surface antigens identifying B cells (Ig) and T cells (Thy-1, Lyt-1,2,3) and macrophages (MAC-1).

The HR assays allow detection of the interactive function of key immune components, including T and B lymphocytes, macrophages, and the complement system (12). For example, resistance to intracellular microorganisms requires induction of CMI through T lymphocyte-macrophage interaction, production of cytokines, and bactericidal activity of macrophages. Resistance to transplantable tumors is dependent on T cell function and natural cytotoxicity.

## Predictive Value of Tier Tests

A study was performed to evaluate each of the tests, and combinations of tests, in the tier battery for immunotoxicity (Table 1) for its ability to predict immunosuppression (6). The database for this analysis consisted of over 50 chemicals that had been tested by the National Institute of Environmental Health Sciences, the Chemical Industry Institute of Toxicology, and the U.S. National Toxicology Program. Individually, two tests were able to predict immunotoxicity in rodents. The test that had the greatest predictive power was enumeration of the lymphocyte populations (based on cell-surface markers, 83% correct predictions), followed by the PFC assay (78% correct). The sensitivity of these tests may be a result of relying on multiple immune processes. The remaining tests in

the battery were not good predictors of immunotoxicity.

Combinations of tests were examined to determine their ability to identify immunotoxic chemicals (6). The combination of the PFC response, or lymphocyte surface marker determination, with almost any other assay increased the ability to detect immunotoxicity to greater than 90%. The combination of PFC and surface markers gave 91% accurate prediction. DTH alone had poor predictability, but when coupled with almost any other test, yielded high predictivity. This suggests that DTH has little functional interdependence with the other tests in the battery (6). The findings of the study suggest that some tests could be deleted from the battery of assays without significantly decreasing the likelihood of identifying a potential immunotoxicant.

A major issue that remains in the use of these tests is that of immune system reserve. Further information is needed about the relationship between qualitative and quantitative changes in immune tests and development of clinical disease. The threshold level in each test must be addressed in considering whether functional changes imply clinical disease.

## Nonhuman Primates

Immunotoxicity tests have been performed using several nonhuman primates including rhesus macaques, cynomolgous monkeys, and marmosets. Most of the assays involve blood or serum. In some models, monoclonal antibodies to human leukocytes can be used (13). Additionally, assessment of DTH lymphocyte proliferation to mitogens, NK function, and cytokine production have been used to assess effects of chronic exposure to Arochlor 1254 (13). It is expected that use of these models will increase with the availability of further biotechnology products.

## Immunotoxicity Assessment in Humans

There are severe limitations to assessing immune changes in humans. Noninvasive tests must be used. Additional difficulties include establishment of the exposure dose, and the continual background of environmental exposures. Numerous factors contribute additional heterogeneity in responses. Immunological parameters are affected by sex, gender, race, stress, medications, and coexisting disease.

Testing schemes for preliminary evaluation of individuals exposed to immunotoxicants have been proposed by several

organizations and agencies including the World Health Organization, U.S. Centers for Disease Control and Agency for Toxic Substances and Disease Registry, and the U.S. National Research Council (13). Common features of the approaches are inclusion of blood cell and differential counts, assessment of lymphocyte phenotypes, determination of immunoglobulin levels or an antibody response, and assessment of autoantibodies or IgE. Additional tests recommended by some of the agencies include an assay for DTH and a measurement of nonspecific immunity, such as NK activity or phagocytosis.

Interpretation of immunotoxicity test results in humans is usually difficult. The variation in human responses is great, frequently exceeding a coefficient of variation greater than 20 to 30% (13). It is recommended that a positive diagnosis be based on a pattern of changes, rather than on the results of any one test (13).

## Animal Models of Host Resistance

Host resistance models detect the effects of chemicals on the functional integrity of the immune system. Ideally, the model should simulate a prevalent human disease, for example, viral pneumonia. The test animals should be infected with a low dose of infectious agent and by a natural route, to avoid overwhelming the host immune system (12). Mice are typically used because of favorable cost, convenience, and knowledge of immune system components and interactions. Rarely is a single cell type solely responsible for HR. Numerous humoral and cellular responses contribute to defense against microorganisms including antibody production by B cells, cytotoxic T cells, NK cells, neutrophils, and macrophages. Although there is redundancy in the immune system such that many components contribute to HR, some are more important for particular infectious agents than others. Several protozoan parasites effectively stimulate antibody synthesis, thus diverting the immune system from a protective cellular response.

Both immunosuppression and immunoenhancement can be detected in HR models (12). A list of these models is provided in Table 2. These assays are usually performed by challenging the chemically dosed animal with either an infectious agent or a tumor. Challenge with extracellular microorganisms, such as *Salmonella typhimurium*, stimulates interaction of T cells, B cells, and macrophages to produce

**Table 2.** Models of host resistance.

Microorganism	Immune response
<i>Listeria monocytogenes</i>	Delayed hypersensitivity; protection by macrophages and T cells
<i>Streptococcus pneumoniae</i>	Acute pneumonia; protection by antibody and complement
<i>Plasmodium yoelii</i>	Elimination of the parasite requires B cells, T cells, and macrophages
Syngeneic tumor cells (B16 F10, melanoma) (PYB6, carcinoma)	T cells and NK cells needed to resist tumor growth
<i>Trichinella spiralis</i>	Inflammation, expulsion of parasite; T cells required, antibodies also involved
Influenza virus	Mortality; antibody, interferon required
Murine cytomegalovirus	Resistance to virus proliferation; antibody, macrophages, NK cells required

specific antibodies. Resistance to intracellular organisms such as *Listeria monocytogenes* stimulates induction of CMI through T cell and macrophage interaction with production of cytokines. Resistance to transplantable tumors requires functional activity of T cells and NK cells.

### Tests That Correlate with Host Resistance

Host resistance assesses the function of the immune system *in toto* and from a risk assessment point of view is considered to be the most relevant test of immunotoxicity. The immune responses that contribute to HR include humoral antibody production by B cells, cytotoxic T cells, NK cells, neutrophils, and macrophages. Both immunoenhancement and immunosuppression can be detected with HR assays. Insignificant changes in a number of immune parameters may together result in immunosuppression that lowers HR.

An attempt was made to develop a mathematical model that would explain the relationship between immune tests and HR assays (12). The chemical selected was cyclophosphamide, a well-recognized immunotoxicant. Most of the immune function tests showed a good correlation with HR assays. There were no instances where HR was altered without effects seen in immune tests. However, there were cases of immune test changes without effects on HR. No single test was fully predictive for HR. The best association was found with DTH, whereas leukocyte counts and lymphoproliferative response were poor correlates of HR. Concordance was increased with combinations of tests. Pairwise combinations of PFC, surface markers, and DTH gave greatest concordance.

Results from most of the immune function tests demonstrated a linear relationship with those from HR assays. A linear dose response implies that a slight change in the immune test would reflect a change in HR.

A linear quadratic response would follow more closely a threshold model for risk at low doses. Included in this category was the surface marker determination. More analyses such as these are needed to provide insight into the relationship between assays and HR. Such efforts would reduce the need to perform many of the tests, while improving the quantitative estimate of immune risk from chemical agents.

### Assessment of Hypersensitivity

Hypersensitivity is a heightened immunologic response that causes tissue disturbance, disruption, or death. The response requires at least two exposures to the causative agent. The first exposure primes the system to respond; the second results in the physiologic change or tissue damage.

Hypersensitivity reactions may be mediated by antibody, with the frequent result being a rapid response occurring within minutes of exposure. Alternatively, reactions may be mediated by T lymphocytes, resulting in reactions occurring up to 48 hr following contact with the allergen (14).

The two major hypersensitivity responses to chemicals are contact hypersensitivity and respiratory hypersensitivity. The assays used to assess these responses are listed in Table 3. Contact hypersensitivity is a delayed-type skin reaction characterized by cellular inflammation at the site of exposure. Respiratory hypersensitivity is a response of the respiratory tract. Those responses that occur rapidly are believed to be the result of IgE antibody bound to specific receptors on mast cells and basophils. Reexposure of the sensitized host to a previously encountered allergen results in cross-linking of antibodies by the allergen, degranulation of the cells, and release of biologic mediators. The latter are responsible for the vasodilatation and airway constriction characteristic of respiratory

**Table 3.** Models of chemical hypersensitivity.

Dermal hypersensitivity
Local lymph node assay
Cutaneous basophil hypersensitivity
Guinea pig maximization test
Guinea pig delayed contact hypersensitivity
Mouse ear swelling test
Fluorescence analysis of cell surface markers
Respiratory hypersensitivity
Guinea pig inhalation model
Rat inhalation model
Mouse model

For details of assay procedures, see Burleson et al. (4).

hypersensitivity. Late-onset respiratory responses are recognized by a decrease in FEV<sub>1</sub> (forced expiratory volume in 1 sec) and are associated with tissue inflammation, frequently rich in eosinophils (14). The immunologic mechanism(s) responsible for the respiratory reaction to chemical allergens is largely unknown.

### Dermal Hypersensitivity

A wide variety of chemicals cause dermal (contact) hypersensitivity. Traditionally, guinea pig tests have been used to identify contact allergens; the most widely used test is the guinea pig maximization assay (15). The principle of this test is the elicitation of erythema and edema in previously exposed animals. Variations of the method include intradermal or epicutaneous exposure, occlusion of the test site, and incorporation of adjuvant at first exposure (induction). A major disadvantage of the guinea pig test is the subjective end point requiring visual assessment of erythema. Other problems arise from colored allergens and irritation by the agent.

### Local Lymph Node Assay

This assay measures the induction phase of the sensitization process and is based on the assumption that the proliferative response to first exposure correlates with the sensitizing potential of the chemical (i.e., the response to subsequent exposures) (16). The assay is performed by applying the test material to the ears of CBA/Ca mice on 3 consecutive days. Two days later, <sup>3</sup>H-thymidine is injected intravenously. Mice are sacrificed 5 hr later, and the draining auricular lymph nodes are excised and pooled for each experimental group. Radioactivity is counted in a scintillation counter. The assay is based on the finding that lymphocytes from sensitized mice proliferate *in vitro* when cultured with antigen-presenting cells displaying the relevant antigen on their surfaces. Results

are expressed as total counts or as a stimulation index (comparison of experimental with vehicle-treated animals).

Advantages of this test are its speed and objective end point. Disadvantages are the use of radiolabeled chemicals, and the assumption that induction of an immune response correlates with the elicitation response. The method has undergone validation studies and is appropriate as a first-stage test in the assessment of skin sensitization potential.

### Mouse Ear-Swelling Test

The mouse ear-swelling test (MEST) was designed to identify contact sensitizers by measuring the inflammation associated with the elicitation phase of the response. This assay is based on the findings that delayed-type contact sensitivity could be transferred in the mouse through use of thymus-derived cells.

The test is performed by topical application of the chemical to the abdomen of mice (17). Several days later, animals are challenged on the ear with the chemical; ear thickness is measured 24 and 48 hr later. The increase in thickness when compared to the control ear is a measure of the contact hypersensitivity response. Variations of the method include injection of adjuvant at the induction site, irritation and abrasion of the skin to increase absorption, multiple induction applications of the chemical, and supplementation of the diet with vitamin A prior to testing (18). A linear dose-response effect was demonstrated with a variety of contact sensitizers, including isocyanates and formaldehyde (19).

Strengths of the test when compared with traditional guinea pig tests are lower cost, shorter duration of each test, less vivarium space required, a more objective end point, and the ability to monitor responses of colored materials. The test has excellent specificity but low sensitivity (17). Difficulty may be encountered in interpreting the response if fluid collects in the ear. This test is appropriate as a first-stage test for the skin sensitization potential of chemicals.

### Fluorescent Analysis of Cell Surface Markers

This method evaluates the surface antigens of the mononuclear cells associated with the contact hypersensitivity lesion. In addition to surface markers, cell cycle and activation states can be assessed through the use of specific fluorescent-labeled reagents (20). The advantage of this method is its ability to analyze thousands of cells by several

parameters. Increases in CD4 T lymphocytes were shown following sensitization with dinitrochlorobenzene.

B6C3F<sub>1</sub> mice are sensitized by epicutaneous exposure on several consecutive days near the shoulder blade. Ear challenge is performed on days 14 to 16. The ears are harvested 24 to 48 hr later, and cells dispersed and prepared for staining with reagents that may include L3T4 (CD4), Lyt-2 (CD8), or B220 (CD45RA, specific for B cells). Results have generally mirrored those obtained with MEST analysis. Analyses have indicated that CD4+ cells, but not CD8+ cells, are significantly increased at the elicitation site on days 1 and 2 after challenge. A difference was noted in cells in the lymph nodes following challenge when compared with those in sensitized, nonchallenged animals. Nonchallenged animals demonstrated more CD8+ cells on day 3 when compared with challenged animals.

### Radioisotope Method to Assess Allergic Contact Dermatitis

This method addresses the potential problem with MEST that fluid buildup rather than increased cellularity may be detected. Use of a radioisotopic assay permits measurement of inflammatory cells with increased sensitivity and objectivity. The procedure utilizes B6C3F<sub>1</sub> mice and sensitization is similar to that described for the flow cytometric procedure (21). On day 8, animals are injected in the tail vein with <sup>125</sup>I-BSA and challenged on the ear with the test chemical. Three hours later, ears are biopsied to measure irritancy. For evaluation of ACD, <sup>125</sup>I IUdR is injected after treatment; 48 hr later ears are taken and counted.

Weak sensitizers pose a problem for this assay. Compared with MEST, the method was more sensitive in revealing activity of glutaraldehyde, but less sensitive with 2-mercaptobenzothiazole. Dietary supplementation with vitamin A may increase sensitivity as it has with MEST.

### Respiratory Hypersensitivity

Respiratory hypersensitivity is diagnosed in humans by a decrease in FEV<sub>1</sub> after exposure to the agent (14). For proteins and other high molecular weight materials, the response is believed to be of immunologic origin; for low molecular weight materials such as industrial chemicals the mechanism remains unclear (14).

Mechanistically, responsiveness to high molecular mass allergens is usually attributed to allergen-specific IgE. Animal models have

been developed to better understand the pathogenesis of the disorder and to compare potencies of test agents, largely proteins. As reviewed by the author (22), a variety of species has been utilized including mice, rats, guinea pigs, rabbits, dogs, sheep, horses, and nonhuman primates. Differences among the models include animal species utilized, route of administration of the agent, protocol for induction and elicitation of responses, type of response measured, and judgment of a significant response.

Low molecular weight (LMW) allergens (< 1 kDa) are considered too small to stimulate an immune response, but are capable of this activity when chemically associated with a large molecule, such as serum albumin, as a hapten (14). Models of respiratory sensitization by LMW agents include the guinea pig, mouse and rat models, and the mouse IgE model (22).

### Guinea Pig Model

Guinea pigs have been the species of choice for predictive models of respiratory hypersensitivity. The inhalation model (23) and the intratracheal model (24) rely on the physiologic respiratory response of animals to challenge with the sensitizing agent, as well as on the production of allergic antibody. These models have been calibrated to human sensitization through the use of detergent enzyme allergens, for which there are good human exposure and response data (24).

Guinea pig methods for measurement of chemical-induced respiratory allergy also rely upon the inhalation route for both the induction and elicitation phases of reaction. Elicitation of the response to respiratory challenge is performed with the chemical, or with a protein conjugate of the chemical (23). Less emphasis is placed on the production of allergic antibody, as few chemicals induce such antibodies in humans, and it is uncertain if antibodies have a role in the sensitization response.

The inhalation model was developed for use with toluene diisocyanate (25), and has been applied successfully to the study of other isocyanates and some acid anhydrides. It was not able to detect sensitization to some reactive dyes (23), although allergic antibody was produced to the latter.

Drawbacks of the guinea pig models include the cost of the studies and the technical expertise required to generate and monitor atmospheres of reactive chemical sensitizers and to monitor respiratory responses in challenged animals. These methods were developed

mainly for investigational purposes and not for routine screening.

### Mouse Model

A mouse model has been described to assess the relative allergenicity of proteins and polypeptides (26). The benchmark allergen used to compare responses is the protease, subtilisin. Intranasal and intratracheal dosing have been used. Specific antibodies are measured as a function of the dose of antigen administered, the dosing matrix, and the isotype of antibody produced. Using subtilisin, peak antibody responses were observed after 6 to 8 weeks of dosing. Detergent administered with the allergen augmented the TgG1 responses approximately 4-fold. IgE responses were inconsistent. Differences in potencies of proteins paralleled findings with the guinea pig model. Compared with the guinea pig method, the murine system has the advantage of lower cost, easier handling, and need for less material.

**Mouse IgE Test.** The primary reason for development of the assay was for predictive purposes, rather than for mechanistic studies (27). The method is based on the assumption that respiratory allergens stimulate a Th<sub>2</sub> lymphocyte response in mice, whereas contact allergens would provoke a Th<sub>1</sub> response. Th<sub>1</sub> cells secrete IL-2 and interferon  $\gamma$ , whereas Th<sub>2</sub> cells secrete IL-4 and IL-10. The latter cytokines stimulate production of IgE.

The assay is performed by repeatedly exposing mice epicutaneously to the chemical; usually three concentrations are used. After an interval of several weeks, serum is taken for evaluation of IgE and cytokines.

This method remains to be validated by independent laboratories. Potential advantages of the method are cost and speed. Because nonspecific IgE is measured, there is no need to prepare hapten conjugates for detection of specific antibodies.

### The Rat Model

A rat model assesses chemical allergens by measurement of airway inflammation (28). Animals are exposed via nose-only inhalation to the chemical for 1 or 2 consecutive weeks. Histologic assessment is made of respiratory tissue. Findings have included eosinophilic inflammation, secretory cell hyperplasia, epithelial desquamation, mucus plugs and cellular debris in the airway lumen, and smooth muscle hypertrophy. Regression of disease is monitored by physiologic responses during an observation period of 1 to 2 months after cessation of exposure.

For all models, validation is needed using both positive and negative chemicals. Moreover, the sensitivity of the methods in comparison with human data must be addressed.

### Assessment of Autoimmunity

Autoimmune disease represents a significant clinical problem in that approximately 5% of the U.S. population is affected. Moreover, as the population ages, the likelihood of an increase in prevalence is expected. Genetics plays a large role in susceptibility to autoimmune disease, and environmental factors influence disease development. Infection is the most frequently cited environmental agent. Physical factors such as ionizing radiation and sunlight have been cited, as have therapeutic drugs. Penicillamine has been associated with cases of myasthenia gravis.

Animal models have been described for experimental induction of autoimmune disease (29). However, these models are used mainly to elucidate the pathogenesis of autoimmunity rather than for toxicologic assessment.

There are several mechanisms associated with autoimmunity (29). Release of antigens that are normally segregated from the immune system, for example, the lens of the eye, can initiate the process. A second mechanism relies on molecular mimicry. This process is based on the observation that antigenic determinants in the body are often replicated on foreign antigens. Beta hemolytic streptococci possess membrane determinants that resemble antigens of the cardiac myocyte. Molecular mimicry may also occur when changes are induced on endogenous antigens. Altered self-molecules are partially foreign and may be recognized as foreign by the immune system. Viruses may attack the reticuloendothelial system and alter immune recognition or regulation of cell populations. It must be emphasized, however, that although autoantibodies may be produced, they do not imply disease (29).

### Risk Assessment

The guinea pig intratracheal model has been used for assessment of risk for respiratory hypersensitivity from protein allergens (30). For LMW chemicals, hazard identification can involve structure-activity relationship (SAR) analyses or assessment of electrophilicity such as the ability of the chemical to haptenate a protein or peptide. For either, determination of specific antibody production after injection into animals has also been used for hazard identification.

Dose-response data can be obtained from the guinea pig models. Calibrations of the animal model to human response are performed using benchmark chemicals for which there is both human and animal data (24). One example is hypersensitivity to the protease subtilisin. Antibody titers and pulmonary responses to atmospheric levels of this enzyme are known for animals and for humans. In both, specific antibody is produced at allergen concentrations lower than those required for induction of pulmonary sensitivity.

For LMW allergenic chemicals, benchmark chemicals for which there are guinea pig and human data of sensitization are toluene diisocyanate and trimellitic anhydride (24). Because the U.S. threshold limit value (U.S. TLV) for each chemical appears to protect against sensitization, dose-response data obtained from animals can be compared with the human TLV to calibrate the two systems.

Risk assessment for immunosuppression should be subjected to the same principles as that for other disciplines. Toxicokinetics should be studied to evaluate exposure levels obtained in animals and relate this to expected human levels. The rat and mouse tier systems were adequate in identifying the immunotoxic hazard of azathioprine and cyclosporine, compounds that are potent immunomodulators in rodents and humans (13). The problem is difficult for compounds that exert subtle effects in rodents. In such cases, HR assays will gain importance. However, no single HR model will suffice as each model requires different components of the immune system to work in concert. It is of great importance to know whether increases in host susceptibility to immune challenges follow a thresholdlike or a nonthreshold model as a function of immunosuppression (13). Additionally, immune reserve may be applicable to individuals, but in populations where immunocompetence may be compromised because of chronic disease, immunoreserve may not be applicable.

### Expected Advances

The multicomponent nature of the immune system has driven the development of numerous tests to assess its individual components as well as its integrated functioning. With the current focus on reduction and replacement of animal use in toxicology, alternative methods to standard mammalian models are emerging to assess immune status after chemical exposure. There is a trend toward the use of fewer



**Table 4.** Future developments in assessment of immunotoxicity.

<i>In vivo</i> models
Use of fewer, but more predictive tests
Histopathology: gene activation, DNA damage, <i>in situ</i> hybridization, magnetic resonance imaging to detect chronic effects
Lymphoid organs: popliteal lymph node assay, bone marrow cell assays
Flow cytometry for cell enumeration and functional assessment
Use of feral animal models, i.e., fish
<i>In vitro</i> systems
Hapten-protein association assays
Cell cultures: cytokine assays
Computational structure-activity models
Mechanistic and predictive models

but more predictive tests (Table 4; section on Predictive Value of Tier Tests).

New animal tests are being developed as screening assays to detect autoimmunogenic chemicals, most frequently in drug assessment. One promising method is the popliteal lymph node assay (16) which can be performed in mice and rats. The assay measures activation of the draining lymph node of the hind paw after injection of the test agent into the hind foot pad. The method has been able to detect pharmaceuticals prone to induce systemic autoimmune-like reactions in an early stage of preclinical testing. The method requires validation.

## Fish Models

The use of nonrodent species for immunotoxicologic evaluation of chemicals has gained attention. Fish models have been described to assess the effects of environmental pollutants on the immune system (31). Fish do not have bone marrow or lymph nodes, but possess hematopoietic tissues functionally equivalent to that of mammals. Their circulating white blood cells are capable of phagocytosis and of rejecting allografts. Fish can exhibit inflammation and hypersensitivity responses, synthesize specific antibody, respond to mitogens, elicit a MLR, process and present antigens, and release biologic mediators such as interleukins, cytokines and arachidonic acid metabolites (31). In addition, fish demonstrate immunologic tolerance and memory (more rapid and stronger response to a second contact with antigen). Chemicals that are immunotoxic in mammals and also immunotoxicants in fish are lead, nickel, organochlorine and organophosphates, pesticides, halogenated aromatic hydrocarbons, and polycyclic aromatic hydrocarbons (31).

Advantages of fish systems include their large size (resulting in plentiful cells), easy maintenance in the laboratory, and low cost. Disadvantages are dosing regimens, and current limited knowledge of the immune tissues and mediators. Data are needed to evaluate the sensitivity of the fish immune system compared to that of humans.

## Computational Toxicology Methodology

Several *in vitro* and *in vivo* models have been developed for predictive testing of sensitizers. For respiratory chemical sensitizers, focus has been on the ability of the chemical to bind to a protein. Shortcomings with this methodology are that the procedures identify electrophiles and nucleophiles, but not all sensitizers belong to these classes (32). Further, not all electrophiles and nucleophiles are sensitizers. Thus, such tests are neither sufficiently sensitive nor specific for predictive purposes. SAR models have been reported for both respiratory and dermal chemical sensitizers (33). For contact sensitivity, several SAR models have accurately predicted strong-moderate sensitizers by identifying electrophiles, nucleophiles, proelectrophiles, and pronucleophiles (34). The CASE/MultiCASE artificial intelligence system has moderate success in identifying weak sensitizers (34). This latter system differs from others in that it does not assume a mechanism of sensitization, but rather operates from identification of structural fragments that have a statistical association with the class of sensitizing chemicals as compared with non-sensitizing chemicals.

An SAR model for respiratory chemical sensitizers has been described (2,33). The data base consists of chemicals identified from human case reports. The model has identified chemical fragments associated with activity. The model is expected to enable predictions of respiratory sensitizing capacity of chemicals, as well as provide mechanistic insight into the process of sensitization.

Current investigations employing computational models are considering chemical fragments and physical-chemical features of chemicals, as well as their metabolic conversions, for predictive purposes. It is anticipated that future SAR models will tackle the problem of predicting immunotoxicity from exposure to mixtures of chemicals.

## Molecular Methodology

Methods for identification of genes and regulatory elements in cells are expected to

aid in identification of susceptible individuals. Such tools will also identify cell activation stages, and markers of oxidative stress. Identification of genes involved in asthma and allergy will guide mechanistic studies, as well as have application to the development of drugs and other approaches for treatment.

## Biomarkers

Cytokines are glycoproteins that are produced and secreted by cells in response to cellular activation. Their production is regulated at the transcriptional and post-transcriptional levels. They appear to be a central factor governing the induction and regulation of immune responses and have considerable potential as biomarkers of immune toxicity. Further epidemiologic studies are needed to clarify the usefulness of biomarkers for detecting immunotoxicity and health outcomes.

## Validation

There must be validation of the tests that evaluate immunotoxicity. The goal of validation is to determine which methods should be recommended in the testing guidelines of regulatory agencies. Validation consists of testing a set of compounds with known positive and negative immunotoxicity and determining the sensitivity, specificity, accuracy, and precision of the tests (13). These parameters are defined as sensitivity, the ability to identify positive chemicals; specificity, the ability to identify negative chemicals; accuracy, the ability to measure the intended end point; and precision, the ability to reproduce results. Some test methods have undergone validation as described in previous sections ("Predictive Value of Tier Tests" and "Risk Assessment"). However, consideration must be given to the question of whether validation requires compounds to be shown to be immunosuppressive in humans as well as in rodents.

## Conclusions

Trends are emerging among the newer methods being developed for immunotoxicity testing. Prominent among the trends is less use of whole animals, more *in vitro* tests, more use of computational methods, and greater interest in *in vitro* mechanistic assays. As progress is made in understanding the regulatory relationship among immune system components and in gaining mechanistic understanding of immunotoxicity, greater use of cell number determinations, cell surface and functional antigens, and simple focused assays is predicted.

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